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Development and Validation of a Novel Bioanalytical Method for Estimating Epigallocatechin 3 Gallate in Wistar Rat Plasma by RP-HPLC Employing Gradient Elution Techniques

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ABSTRACT: The goal of this study was to provide a new, easy, accurate, cost-effective, exact, sensitive, specific, robust, and rugged method for quantifying Epigallocatechin 3 gallate in wistar rat plasma using reverse phase high-performance chromatography (RP-HPLC). The stationary phase was a Zorbax SB C18 5µ (4.6*150) mm column, while the mobile phase was water with 0.1 percent formic acid (A) and acetonitrile (ACN) with 0.08 percent formic acid (B). The experiment was conducted at 30°C with a flow rate of 1.0 ml/min with PDA detectors at 274 nm. With an r^2 of 0.9999, the method was shown to be linear in the concentration range of 0.2–25 µg/ml. At the same retention time (Rt) of epigallocatechin 3 gallate, no interference of co-eluting peaks of endogenous chemicals from the biological matrix was found. The intraday and interday precision RSD (%) was found to be within acceptable limits (less than 2%). The overall mean recovery percentage was determined to be 96.92 %. The LOD and LOQ were determined to be 0.0682 ± 0.0011 µg/ml and 0.205 ± 0.004 µg/ml, respectively. In short-term and long-term stability tests, auto sampler, bench-top, and freeze-thaw stability tests were found to be stable. The developed approach reported was determined to be well within acceptable limits. As a result, in the future, this method can be successfully employed in clinical laboratories to estimate epigallocatechin 3 gallate alone or in conjunction with other analytes or markers in pharmacokinetic, bioequivalence, and therapeutic drug monitoring.

KEYWORDS: Epigallocatechin 3 gallate; Wistar rat plasma; RP-HPLC; gradient elution; bioanalytical method; validation.

1. INTRODUCTION

Green tea is another name for *Camellia sinensis*. Due to its stimulating properties, alluring aroma, refreshing flavour, and numerous health advantages, tea is one of the most popular non-alcoholic drinks and is eaten by more than one third of the world's population. There are lots of polyphenols, flavonoids, and alkaloids in *Camellia sinensis* (family: *Theaceae*). Green tea extracts provide a variety of health benefits, many of which are based on the presence of polyphenols and their derivatives. According to a variety of studies, catechins and alkaloids are the two major classes of phytoconstituents discovered in *Camellia sinensis* leaves. Epigallocatechin-3-gallate, the most active polyphenol, is found in unfermented *Camellia sinensis* leaves. Epigallocatechin-3-gallate makes up 10%–50% of all catechin components in *Camellia sinensis* plant leaves. In fermented *Camellia sinensis* leaves, epigallocatechin predominates, while epigallocatechin-3-gallate is less prevalent [1]. The primary and most important tea catechin, (-)-epigallocatechin gallate, is assumed to be in

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charge of most of the biological effects of green tea. The ester of gallic acid with epigallocatechin is EGCG. When EGCG is present in its purest form, it appears as an odorless, white, pale pink, or cream-colored powder or crystal. Water (5 mg/mL clear, colourless solution), acetone, ethanol, methanol, pyridine, and tetrahydrofuran are among the solvents in which it is soluble. Its molar mass is 458.372 g/mol, and its chemical formula is $C_{22}H_{18}O_{11}$ [2]. Its melting point is between 140 and 142 °C. There are several names for epigallocatechin gallate, including epigallocatechin 3-monogallate, epigallocatechin 3-gallate, (-)-epigallocatechin-3 gallate, (-)-epigallocatechol gallate, EGCG, and (-)-epigallocatechin-3-O-gallate. Numerous in vitro and in vivo preclinical studies, as well as clinical trials, have demonstrated that the EGCG compound possesses a wide range of biological and pharmacological properties, such as antimicrobial, anti-carcinogenic (anti-tumorigenic), anti-oxidative, anti-allergic, anti-cardiovascular, anti-diabetic, anti-inflammatory, anti-hypercholesterolaemic (lipid clearance), anti-atherosclerosis, anti-mutagenic, anti-aging, decreased risk of osteoporosis, neuroprotective, and immunomodulatory effects. It's interesting to note that studies have shown that EGCG at greater doses might have negative effects on health, mostly through causing hepatotoxicity in both humans and animals. In animal experiments, oral administration of EGCG in aqueous solutions results in poor bioavailability because of rapid degradation. Despite having a low absorption rate, EGCG is quickly transported throughout the body and/or transformed into metabolites. It is broken down by the biotransformation metabolic pathways of methylation, glucuronidation, sulfation, and ring fission. They can also traverse the blood-brain barrier to enter the brain. Bile is the main excretion method for EGCG [1-5].

Gradient elution is recommended for samples containing giant molecules, models with a broad retention time range, samples containing late-eluting interferences that can block the column or cause chromatograms to overlap, and materials dissolved in a weak solvent in a dilute solution. Some samples were separated using isocratic chromatographic elution with a retention duration of the band of interest greater than 0.2 and less than 20 minutes. However, late-eluting interferences pollute the column or interfere with subsequent separations [5-6]. When it comes to herbal extracts, there are numerous phytoconstituents present. Because of the extensive range of retention durations, it is only sometimes possible to separate all phytoconstituents using the isocratic elution approach. An isocratic chromatographic elution separation method requires the quantification of a single chemical, but subsequent interference bands elute endlessly. The difficulty could be solved by using gradient elution, affecting the final bands' quick elution before injecting the following sample. Because of the buildup of strongly retained chemicals on the column, the first use of isocratic chromatographic elution for these samples resulted in rapid loss of column activity and poor separation. Gradient elution, not isocratic separation, eliminated tightly bound material in every run, removing this challenge. As a result, gradient elution was preferred over isocratic elution for separating more complex mixtures, such as phytoconstituents, with excellent peak resolution and a short retention period [7-9].

Using capillary electrophoresis (C.E.) and high-performance liquid chromatography (HPLC), extensive investigations on detecting catechin and caffeine in many varieties of *Camellia sinensis* plant leaves have been described [6–12]. Also, research has been done on spectrophotometry [13], ultra-performance liquid chromatography (UPLC) [14, 15], reverse phase RP-HPLC [16, 17], ultra-performance liquid chromatography-time of flight mass spectrometry (UPLC-TOF-MS) [18], and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [14].

Even though there are numerous methods for estimating epigallocatechin-3-gallate alone or in combination with other drugs or extracts, no bioanalytical method has been developed and validated using a gradient elution RP-HPLC method for estimating epigallocatechin-3-gallate from Wistar rat plasma. So, the goal of this study was to create and test a new bioanalytical method for measuring the amount of epigallocatechin-3-gallate in the blood plasma of Wistar rats that was easy, economical, accurate, precise, sensitive, strong, and durable.

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2. RESULT AND DISCUSSION

2.1. Linearity

Rat plasma chromatograms, (Low quality control) LQC, (Medium quality control) MQC, (High quality control) HQC, linearity overlay spectra, and a calibration curve for epigallocatechin 3 gallate in Wistar rat plasma are shown in Figures 1, 2, 3, 4, 5, and 6. The linearity results are further illustrated in Tables 1 and 2. The linearity curve for epigallocatechin 3 gallate was determined to be linear utilizing HPLC equipment for concentrations ranging from 0.2 to 25 µg/ml. 0.9999 was found to be the linearity correlation coefficient. The linearity equation for plasma was discovered to be Y = 44929.71 X – 4900.50. The plasma calibration curves for epigallocatechin 3 gallate showed complete linearity and a substantial correlation value. The calibration equation was used to quantify each linearity and quality control sample, and the formed standard deviation was less than 1%. It was discovered that the percent RSD was less than 2%. The accuracy ranged between 96.5 and 110 percent [19].

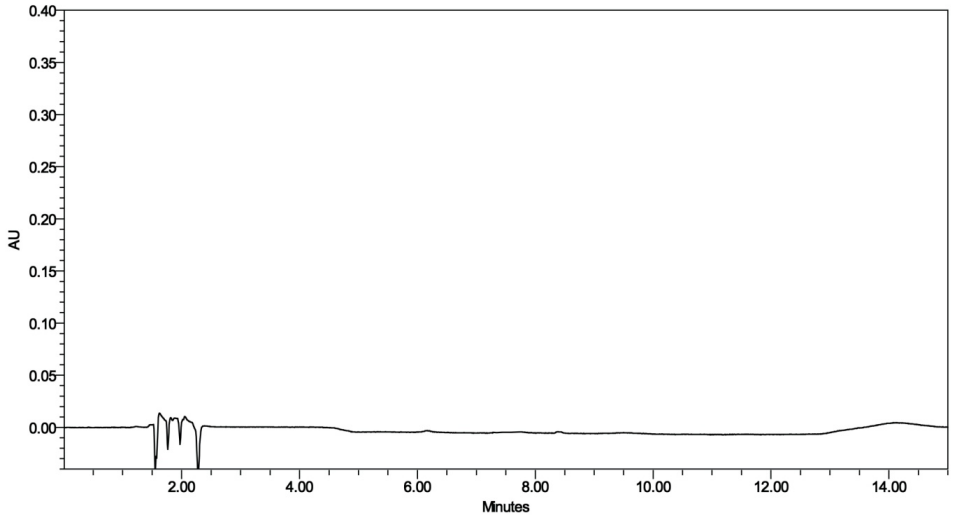

Figure 1. Chromatogram of extracted blank rat plasma

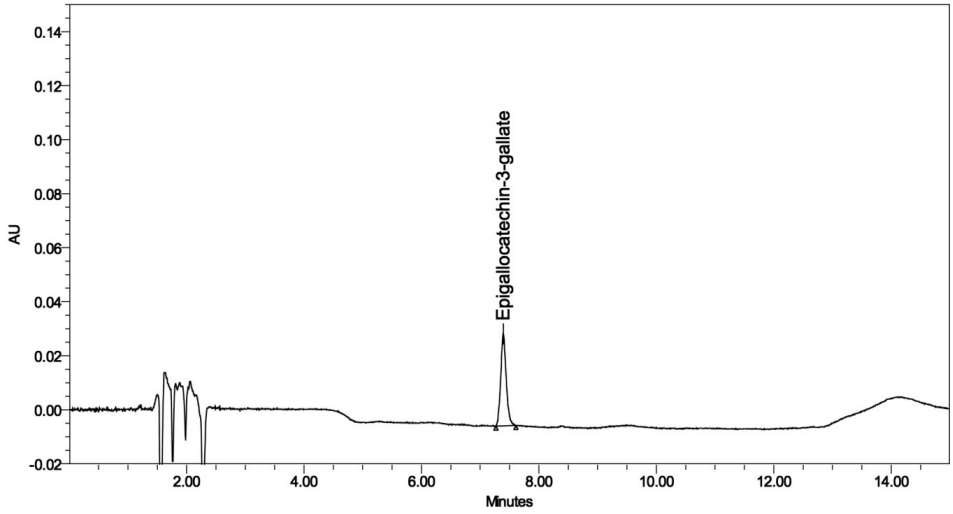

Figure 2. Chromatogram of standard epigallocatechin 3 gallate at LQC level (5µg/ml) spiked in rat plasma. (Rt = 7.417 minutes)

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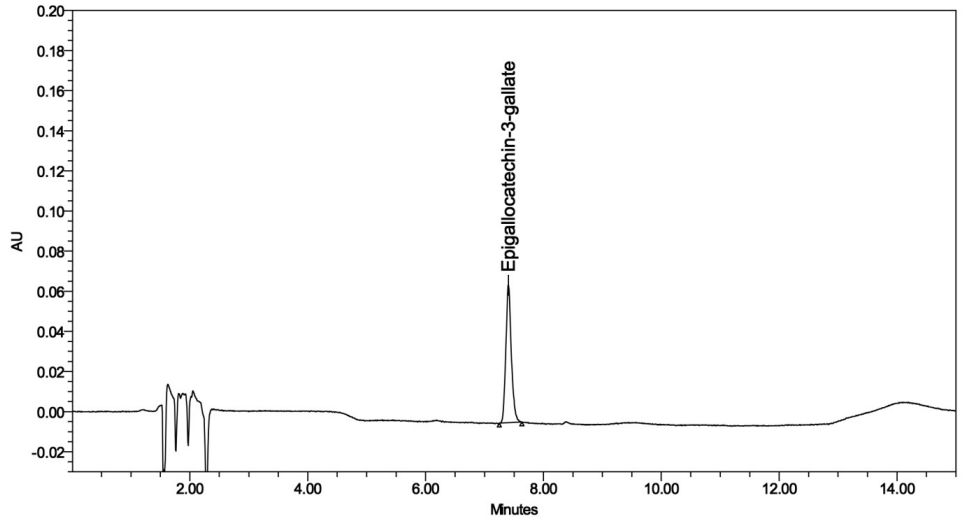

Figure 3. Chromatogram of standard epigallocatechin 3 gallate at MQC level (10µg/ml) spiked in rat plasma. (Rt = 7.417 minutes)

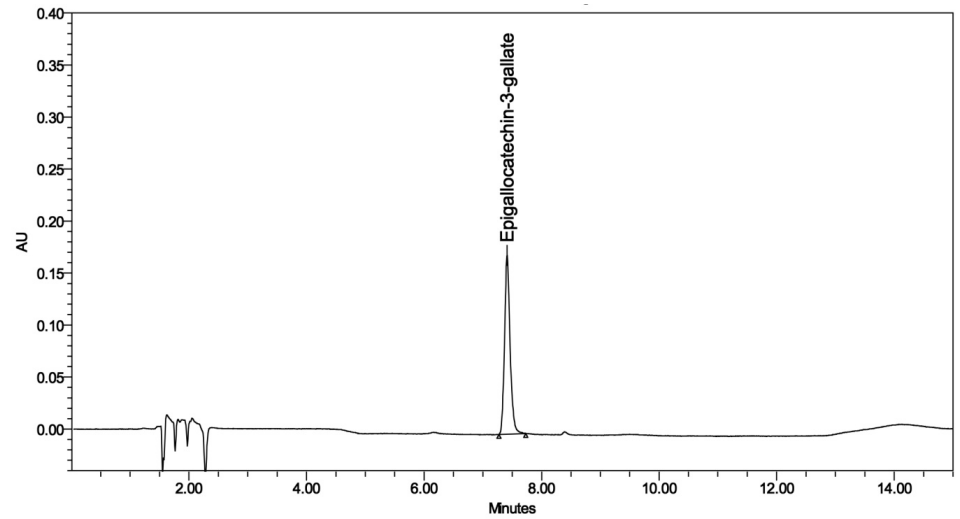

Figure 4. Chromatogram of Standard Epigallocatechin 3 gallate at HQC level (25µg/ml) spiked in rat plasma. (Rt = 7.417 Minutes)

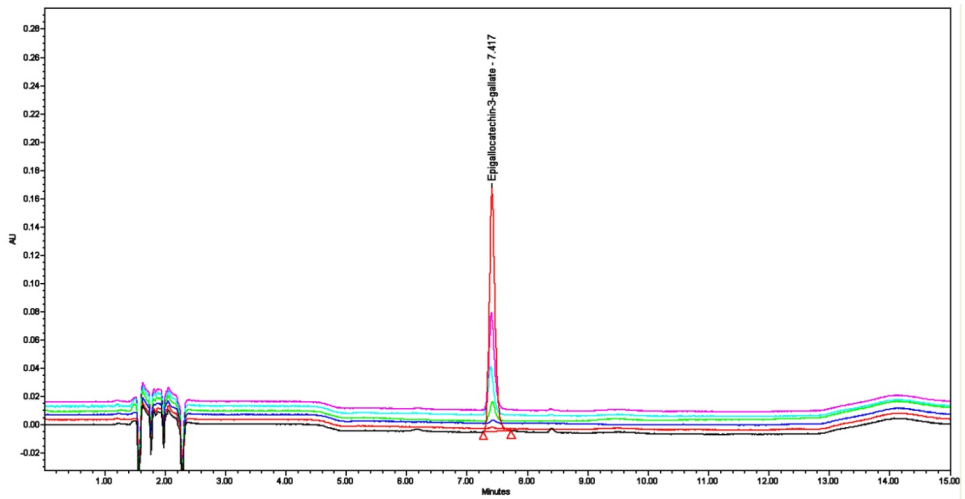

Figure 5. Epigallocatechin 3 gallate spectra in rat plasma with linearity overlay

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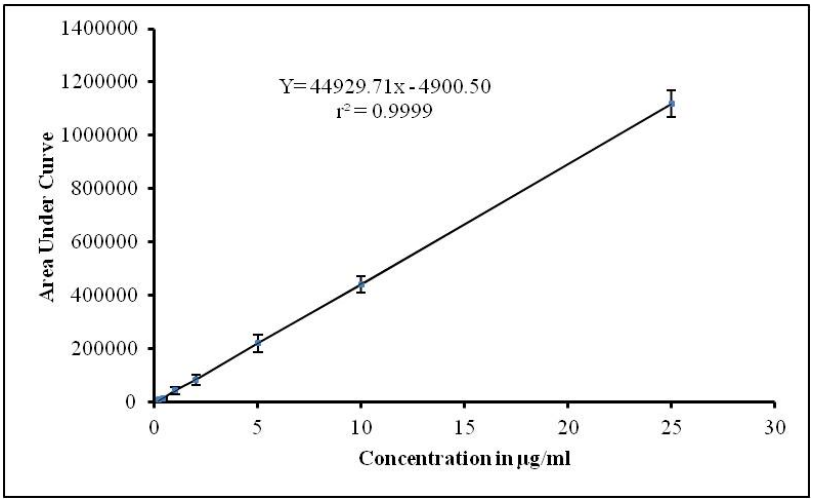

Figure 6. Linearity curve of epigallocatechin 3 gallate in rat plasma

Table 1. Various constants for calibration curve of Epigallocatechin 3 gallate in rat plasma

Parameter	**Value**
Beer's Low Limit	0.5 – 25 µg/ml
Correlation coefficient*	0.9999
Intercept*	-4900.50
Slop*	44929.71
Regression Equation	Y = 44929.71 X – 4900.50
Retention time	7.417 Minutes

* Average of three determinations.

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Table 2. Epigallocatechin 3 gallate linearity in rat plasma

Spiked plasma concentration (µg/ml)	Measured concentration (n=3)(µg/ml)(Mean ± SD	RSD (%)	Accuracy (%)
0.2	0.22 ± 0.003	1.197	110.0
0.4	0.39 ± 0.005	1.315	97.50
1	1.044 ± 0.011	1.135	104.4
2	1.93 ± 0.036	1.876	96.50
5	4.99 ± 0.080	1.603	99.80
10	9.91 ± 0.183	1.841	99.10
25	25.03 ± 0.320	1.278	100.12

2.2. Specificity or selectivity

The chromatograms of blank rat plasma and epigallocatechin 3 gallate spiked plasma samples were compared to test the specificity and selectivity of the new bioanalytical RP- HPLC technology. At three Q.C. levels, blank plasma and epigallocatechin 3 gallate-treated plasma were shown in Figures 1, 2, 3, and 4. After comparing the peaks, it was determined that there were no disturbing peaks at the epigallocatechin 3 gallate retention time (Rt= 7.417 minutes).

The specificity of epigallocatechin 3 gallate was investigated by adjusting the mobile phase composition's gradient slope in the HPLC method. As a result, no interference of co-eluting peaks of endogenous chemicals from the biological matrix was observed during the retention period of epigallocatechin 3 gallate. Furthermore, the previous observation and chromatogram indicated that the co-eluting peak from rat plasma did not interfere with epigallocatechin 3 gallate. As a result, the method developed for detecting and analyzing epigallocatechin 3 gallate in plasma showed specific [19-20].

2.3. Precision and accuracy

The findings of intraday and interday precision and accuracy testing of epigallocatechin 3 gallate in rat plasma using the recommended bioanalytical method are shown in Tables 3 and 4. Intraday percent RSD was less than 2%, with accuracy ranging from 92.5% to 100.28%. The variation in percentage RSD across days was less than 2%, and accuracy was found to be from 92% to 99.68%. The results showed that the suggested bioanalytical technique is accurate, precise, repeatable, and dependable [20-21].

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Table 3. Intraday accuracy and precision data of epigallocatechin 3 gallate in rat plasma

Spiked plasma concentration (µg/ml)	Measured concentration (µg/ml) (n=5) (Mean ± S.D.)	RSD (%)	Accuracy (%)
Morning			
0.2 (LLOQ)	0.193 ± 0.004	1.881	96.50
5 (LQC)	4.970 ± 0.017	0.338	99.40
10 (MQC)	9.962 ± 0.150	1.503	99.62
25 (HQC)	25.070 ± 0.097	0.387	100.28
Afternoon			
0.2 (LLOQ)	0.185 ± 0.003	1.412	92.5
5 (LQC)	4.836 ± 0.054	1.119	96.72
10 (MQC)	9.948 ± 0.099	0.994	99.48
25 (HQC)	24.89 ± 0.061	0.243	99.56

Table 4. Interday accuracy and precision data of epigallocatechin 3 gallate in rat plasma

Spiked plasma concentration (µg/ml)	Measured concentration (µg/ml) (n=5) (Mean ± S.D.)	RSD (%)	Accuracy (%)
First Day			
0.2 (LLOQ)	0.193 ± 0.004	1.881	96.50
5 (LQC)	4.970 ± 0.017	0.338	99.40
10 (MQC)	9.962 ± 0.150	1.503	99.62
25 (HQC)	24.92 ± 0.302	1.211	99.68
Second Day			
0.2 (LLOQ)	0.184 ± 0.002	0.955	92.00
5 (LQC)	4.816 ± 0.071	1.465	96.32
10 (MQC)	9.788 ± 0.086	0.883	97.88
25 (HQC)	24.79 ± 0.131	0.529	99.16

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2.4. Recovery study

The peak areas of epigallocatechin 3 gallate with (plasma) and without biomatrix (solvent) were compared to compute epigallocatechin 3 gallate recovery. The results of the recovery trial are shown in Table 5. Three replicates of epigallocatechin 3 gallate recoveries were examined at three different quality control concentrations: (HQC-25 µg/ml), (MQC-10 µg/ml), and (LQC-5 µg/ml), yielding 98.70 percent, 96.41 percent, and 95.64 %, respectively. The overall recovery rate of epigallocatechin 3 gallate was reported to be 96.92 %. The total % RSD was found to be 1.642 %. Endogenous chemicals can hamper drug analysis in biological samples in biomatrix. When the data was analyzed, it was observed that the matrix peak did not affect the amount of epigallocatechin 3 gallate in the plasma sample [22-23].

Table 5. Recovery study data of epigallocatechin 3 gallate with and without biomatrix

Replicate Number	LQC (Area) Unextracted	LQC (Area) Extracted	MQC (Area) Unextracted	MQC (Area) Extracted	HQC (Area) Unextracted	HQC (Area) Extracted
	229709	219709	456667	440584	1240167	1224414
	229579	219579	456892	440720	1240389	1224120
	229354	219454	457145	440210	1240570	1224057
Mean	229547	219547	456901	440505	1240375	1224197
SD	179.6	127.5	239.1	264.1	201.8	190.5
% RSD	0.078	0.058	0.052	0.060	0.016	0.015
% Mean recovery	95.64		96.41		98.70	
Overall % Mean recovery	96.92					
Overall SD	1.592					
Overall % RSD	1.642					

2.5. Limit of detection

Then, 0.0682 ± 0.0011 µg/ml was discovered to be the LOD. The new bioanalytical HPLC method is sensitive enough to identify low quantities of epigallocatechin 3 gallate in Wistar rat plasma. RSD was found to be 1.59 percent [24].

2.6. Limit of quantification

The quantification limit was calculated using a signal-to-noise ratio (S/N) of 10:1. The new bioanalytical approach's LOQ was discovered to be 0.205 ± 0.004 µg/ml. As a result, our system detected even small amounts of epigallocatechin 3 gallate in rat plasma. Furthermore, RSD was discovered to be 1.918 percent [25].

2.7. Ruggedness

The results of the epigallocatechin 3 gallate ruggedness research in rat plasma are shown in Table 6. Ruggedness was examined at the LQC and HQC concentration levels to see how different operating conditions

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affected retention time and concentration through peak area. The chromatograms of spiking standard epigallocatechin 3 gallate at the two concentration levels (LQC and HQC) in Wistar rat plasma were examined under varied operating conditions. The retention duration or observed concentrations did not differ significantly. Furthermore, it was found that the percent RSD was less than 2%. As a result, the developed method can endure chromatographic operating conditions such as changing operators, columns, and reagent and chemical sources [25-26].

Table 6. Ruggedness study data of epigallocatechin 3 gallate in rat plasma

Condition of chromatographic operation	Spiked plasma concentration (µg/ml)	Retention time (Min.) (n=3) (Mean ± SD)	RSD (%)	Measured concentration (µg/ml) (n=3)(Mean ± S.D.)	RSD (%)	Accuracy (%)
Operator - 1	5 (LQC)	7.408 ± 0.017	0.225	4.947 ± 0.053	1.066	98.93
	25 (HQC)	7.417 ± 0.009	0.124	24.93 ± 0.050	0.199	99.72
Operator - 2	5 (LQC)	7.426 ± 0.007	0.088	4.960 ± 0.067	1.349	99.20
	25 (HQC)	7.409 ± 0.018	0.242	24.92 ± 0.140	0.560	99.68
Column - 1	5 (LQC)	7.415 ± 0.014	0.183	5.062 ± 0.095	1.880	101.24
	25 (HQC)	7.418 ± 0.018	0.236	25.01 ± 0.042	0.168	100.04
Column - 2	5 (LQC)	7.419 ± 0.019	0.252	4.986 ± 0.078	1.556	99.72
	25 (HQC)	7.413 ± 0.015	0.197	24.98 ± 0.062	0.248	99.92
Source of reagent and chemicals (Merk, Mumbai)	5 (LQC)	7.416 ± 0.009	0.125	4.997 ± 0.085	1.702	99.94
	25 (HQC)	7.413 ± 0.016	0.209	25.03 ± 0.064	0.255	100.12
Source of reagent and chemicals (Qualigens Fine Chemicals, Mumbai)	5 (LQC)	7.406 ± 0.014	0.186	5.035 ± 0.063	1.244	100.7
	25 (HQC)	7.416 ± 0.014	0.189	24.99 ± 0.026	0.104	99.96

2.8. Robustness

The robustness of epigallocatechin 3 gallate in rat plasma is shown in Table 7. It was done by altering the parameters and measuring the peak area to see how they affected retention time and concentration. When flow rate, temperature, and organic mobile phase composition increased, retention duration and measured concentration from peak areas dropped. A one-degree increase in column temperature reduces retention time by one to two degrees. The mobile phase viscosity should be kept as low as possible to maintain an appropriate pressure drop (<160 bar) and a reasonable flow velocity through the column. The thickness of the mobile phase is proportional to the pressure drop, while the viscosity of the solvent and the vapour pressure are greatly influenced by temperature. The density of the mobile phase lowers as the temperature rises, increasing the flow rate. As a result, as the temperature and flow rate increased from normal chromatographic conditions, the retention time and measured concentration from peak areas dropped, and vice versa. When the organic mobile phase was kept at -5 %, the concentration and retention time were also increased. At 5 and 25 µg/ml, the retention time was observed to be reduced from 7.417 to 7.15 and 7.17 minutes with a flow rate of +10

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percent. The percent RSD was less than 2% for all parameter modifications. Compared to traditional chromatographic method settings, no significant effect on retention and concentration was observed. As a result, the established bioanalytical approach can be inferred to be reliable regarding the aforementioned essential criteria. As a result, it can be used in everyday laboratory environments [27].

Table 7. Robustness study data of epigallocatechin 3 gallate in rat plasma

Chromatographic parameter	Spiked plasma concentration (µg/ml)	Retention time (Min.) (n=3) (Mean ± SD	RSD (%)	Measured concentration (µg/ml) (n=3)(Mean ± S.D.)	RSD (%)	Accuracy (%)
Flow rate (-10%)	5	7.775 ± 0.097	1.249	4.969 ± 0.077	1.551	99.38
	25	7.767 ± 0.087	1.127	24.970 ± 0.115	0.459	99.88
Flow rate (+10%)	5	7.152 ± 0.129	1.799	4.633 ± 0.083	1.790	92.66
	25	7.177 ± 0.113	1.568	23.130 ± 0.442	1.912	92.52
Temperature (-10%)	5	7.427 ± 0.029	0.390	4.974 ± 0.019	0.391	99.48
	25	7.431 ± 0.052	0.697	25.030 ± 0.057	0.226	100.12
Temperature (+10%)	5	7.231 ± 0.036	0.503	4.653 ± 0.088	1.888	93.06
	25	7.237 ± 0.028	0.392	23.57 ± 0.469	1.988	94.28
Organic mobile phase (-5%)	5	7.682 ± 0.028	0.370	4.998 ± 0.092	1.835	99.96
	25	7.677 ± 0.050	0.655	24.950 ± 0.267	1.070	99.80
Organic mobile phase (+5%)	5	7.208 ± 0.010	0.141	4.677 ± 0.093	1.988	93.54
	25	7.223 ± 0.050	0.691	23.450 ± 0.464	1.978	93.80

2.9. Epigallocatechin 3 gallate stability in rat plasma

Table 8 summarizes the results of a stability study of epigallocatechin 3 gallate in Wistar rat plasma throughout short, long, autosampler, benchtop, and freeze-thaw periods. The RSD percentage ranged from 0.549 percent (bench top stability, free solution) to 1.914 percent (long-term stability). The percent RSD was determined to be less than 2% of the genuine value in all stability studies. No significant degradation of epigallocatechin 3 gallate was observed when studies were conducted at different types of stability. In addition, Epigallocatechin 3 gallate in rat plasma was found to be stable under various storage settings, according to the results of a stability study [25-28].

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Table 8. Stability study of epigallocatechin 3 gallate in Wistar rat plasma

Stability type	Spiked plasma concentration (µg/ml)	Measured concentration (µg/ml) (n=3)(Mean ± S.D.)	RSD (%)	Accuracy (%)
Short term stability (6 h at 30°c)	5	4.913 ± 0.088	1.786	98.26
	25	24.48 ± 0.358	1.464	97.92
Long term stability (10 days at 30°c)	5	4.742 ± 0.088	1.851	94.84
	25	24.11 ± 0.462	1.914	96.44
Autosampler stability (24 h at 5°c)	5	4.707 ± 0.089	1.908	94.14
	25	23.690 ± 0.445	1.879	94.76
Benchtop stability (Old solution)	5	4.688 ± 0.087	1.855	93.76
	25	23.42 ± 0.417	1.781	93.68
Benchtop stability (Fresh solution)	5	5.069 ± 0.064	1.257	101.38
	25	25.03 ± 0.138	0.549	100.12
Freeze-thaw stability (cycle 3, 4 h at -30 °c)	5	4.622 ± 0.084	1.817	92.44
	25	23.26 ± 0.368	1.582	93.04

3. CONCLUSION

This research aimed to develop and validate a bioanalytical RP-HPLC approach for determining epigallocatechin 3 gallate levels in Wistar rat plasma. The new bioanalytical RP-HPLC method has been demonstrated to be buffer-free, simple, precise, accurate, sensitive, rugged, durable, and highly repeatable. ICH rules tested the proposed approach, and the results were deemed satisfactory. There was no evidence of a matrix effect on retention time and measured concentration when the chromatographic parameters and operating conditions were varied consistently. According to the stability investigation, epigallocatechin 3 gallate in rat plasma was stable under various storage conditions and during the sample preparation technique. The new approach was linear between 0.2 and 25 µg/ml, with a higher r^2 of 0.9999. Because of its smaller plasma volume, LLOQ level and detection, accuracy (S.D. & percent RSD found within acceptable range), cost-effectiveness, and simple preparation method, the newly proposed bioanalytical approach is very well suited for estimating epigallocatechin 3 gallate in pharmacokinetics, bioequivalence, and therapeutic medication monitoring studies. This gradient elution chromatographic method can be successfully used to determine epigallocatechin 3 gallate from complex pharmacokinetic samples containing various interfering proteins as well as extracts containing various phytoconstituents with a retention time of fewer than 20 minutes and without the interference of late eluted peaks.

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4. MATERIALS AND METHODS

4.1. Reagents and chemicals

Bangalore-based Sigma-Aldrich provided standard epigallocatechin 3 gallate (EGCG). Merk in Mumbai, India and Qualigens Fine Chemicals, Mumbai, provided HPLC-grade formic acid, methanol, and acetonitrile (ACN).

4.2. Preparation of plasma

Blood was taken from a Wistar rat and placed in a centrifuge tube containing a 5% EDTA solution (Crystal biological solutions, Pune). The blood sample was vortex agitated for one minute before being centrifuged for ten minutes at 5°C at 12,000 rpm. The clear supernatant liquid was separated and kept frozen at -80°C until needed. The Experimental Protocol at Crystal Biological Solutions was approved by the Institutional Animal Ethical Committee (Crystal Biological Solutions Approval No. CRY/2122/070). The institutional and national guides for the care and use of laboratory animals were followed as in "Guide for the Care and Use of Laboratory Animals"

4.3. Preparation of an epigallocatechin 3 gallate standard stock solution

An epigallocatechin 3 gallate standard stock solution was made by weighing 50 mg of pure epigallocatechin 3 gallate and putting it into a volumetric flask with a 50 ml capacity. The mixture was then sonicated for 5 minutes after adding 25 ml methanol. Finally, methanol was used to make up the volume to 50 ml. Whatman No. 41 filter paper [19] filters the produced solution. The concentration of the average stock solution was 1000 µg/ml [28-29].

4.4. Preparation of epigallocatechin 3 gallate working standard solutions

0.1, 0.2, 0.5, 1, 2.5, 5, and 12.5 ml of the standard stock solution (1000 µg/ml) were taken and added to a separate set of 50 ml capacity volumetric flasks, where they were diluted to the 50 ml with methanol to generate concentrations of 2, 4, 10, 20, 50, 100, and 250 µg/ml [30].

4.5. Sample preparation for linearity and quality control

Mixing 2.7 ml blank plasma with 0.3 ml working standing solutions yielded linearity and quality control (Q.C.) samples. For each concentration, 0.3 ml of the standard solution (working) was transferred to different 10 ml Eppendorf tubes, and 2.7 ml of blank plasma was spiked in each working standard solution in Eppendorf tubes. Afterward, each Eppendorf tube was filled with 3 ml methanol and vortex agitated for 1 minute. Each solution was centrifuged for 20 minutes at 5 degrees Celsius at 10000 rpm in a centrifuge machine (Remi, Mumbai). The clear supernatant (3 ml) from each solution was separated and added to new Eppendorf tubes. A 0.42mm membrane filter paper was used to filtrate the clear supernatant before being put into the HPLC equipment in a sequential way. The final linearity quality control samples were prepared at concentrations of 0.2, 0.4, 1, 2, 5, 10, and 25 µg/ml [31-32].

4.6. Optimized chromatographic conditions

Optimized chromatographic conditions and Gradient Chromatographic Method for Epigallocatechin 3 gallate are shown in Table 9 and 10. HPLC (Model: Waters 2695 alliance) was used to determine the amount of epigallocatechin 3 gallate in plasma. A Zorbax SB C18 5µ (4.6*150) mm column was utilized in the bioanalytical technique development experiment. A 0.45 µm Millipore filter was used to filter the mobile phase. The column temperature was maintained at 30 degrees Celsius. The mobile phase flow rate was kept at 1.0 ml/min. The injection volume was 40 µl. At 274 nm, a W2996 PDA-type detector was employed. Throughout the investigation, a chromatographic gradient technique was used. Water containing 0.1 percent formic acid (A) and acetonitrile (ACN) containing 0.08 percent formic acid (B) made up the mobile phase. While the mobile phase components were modified, the flow rate remained unchanged.

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Table 9. Optimized chromatographic conditions

Parameters	**Optimized parameters**
Chromatograph	Waters 2695 alliance
Chromatographic system	Gradient chromatographic method
Column	Zorbax SB C18 5µ (4.6*150)mm
Flow rate	1.0 ml/minute
Temperature	30^{o}c
Type of detector	W2996 PDA
Detection wavelength	274.0 nm
Injection volume	40.00 µl
Mobile phase	Water with 0.1% formic acid: acetonitrile (ACN) with 0.08 % formic acid.
Run time	15 minutes

Table 10. Gradient Chromatographic Method for Epigallocatechin 3 gallate

Time (Minutes)	**%A (Water containing 0.1 percent formic acid)**	**% B (Acetonitrile containing 0.08 percent formic acid)**
0	100	00
2	100	00
10	50	50
11	50	50
11.40	100	00
15	100	00

4.7. Validation

4.7.1. Linearity

Linearity refers to a method's capacity to produce test findings proportional to sample analyte concentration. A set of seven linearity and quality control samples was created by combining 2.7 ml of blank Wistar rat plasma with various volumes of working standard solutions. Before centrifugation, the solution were stirred for 1 minute with a vortex shaker. Eppendorf tubes were used to collect the clear supernatant

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solution. The linearity and quality control samples of epigallocatechin 3 gallate concentrations ranged from 0.2 to 25 µg/ml. Peak area vs. concentration was graphed in three replicates for each concentration sample to obtain the linearity curve. The analyte concentration in each concentration sample was calculated using the regression equation established. The linearity curve was determined to be authentic if all linearity solutions had less than 15% RSD. The correlation coefficient should be greater than 0.96. Epigallocatechin 3 gallate's LLOQ response must be at least three times that of blank plasma [30-33].

4.7.2. Specificity or selectivity

It refers to a method's ability to correctly quantify analyte concentrations even when all other sample components are present. The procedure's precision, accuracy, and linearity are maintained if specificity is assured. Therefore, the first stage in developing and validating an effective strategy is to ensure its specificity. The recommended approach is specific if there is no intervention between co-eluting endogenous component peaks and analyte peaks. The specificity and selectivity of the disclosed method were determined by analyzing the chromatogram of blank Wistar rat plasma without epigallocatechin 3 gallate in three duplicates. Further plasma samples were spiked with epigallocatechin 3 gallate and examined for the intervention of co-eluting peaks in rat plasma chromatograms. The retention time of an epigallocatechin 3 gallate chromatographic peak was discussed. The method's specificity (different gradient slopes) was investigated by changing the HPLC method settings and looking for interference in the chromatogram from endogenous chemicals co-eluting peaks. [32-34].

4.7.3. Accuracy and precision

When a process is reproduced on a homogeneous sample, precision refers to the level of agreement among individual test outputs. Five replicates were tested for intraday (two different times on the same day) and interday (two days apart) precision and accuracy at four different Q.C. levels: HQC (25 µg/ml), MQC (10 µg/ml), LQC (5 µg/ml), and LLOQ (0.2 µg/ml). A regression equation was used to determine how much plasma was recovered. Precision and accuracy were determined as a percentage of RSD and recovery, respectively [30-35].

4.7.4. Recovery study

A recovery study was done to ensure the proposed method's reliability and applicability. It is the degree to which the measured value resembles the actual value. In a recovery study, the efficacy of epigallocatechin 3 gallate extractions in rat plasma samples and the effect of the matrix was studied. Q.C. concentrations of HQC, MQC, and LQC of 25, 10, and 5 µg/ml were used in a recovery investigation. First, three Q.C. concentration levels of epigallocatechin 3 gallate were spiked in blank Wistar rat plasma, and the concentration was determined using the chromatogram of an established HPLC bioanalytical method. Next, the exact three standard concentration solutions were prepared without the biomatrix. Finally, the extraction efficiency of epigallocatechin 3 gallate was determined using the ratio of epigallocatechin 3 gallate concentration with and without biomatrix. Drug recovery does not have to be perfect, but it should be consistent, accurate, and reproducible, according to FDA guidelines [32-36].

4.7.5. Limit of detection

It entails measuring the analyte concentration in a sample containing the smallest amount of analyte; however, it is rarely quantified. The LOD is proportional to the S/N ratio of the chromatographic system. Therefore, the S/N ratio of the analyte should be 3:1 to identify the tiniest amount of analyte. The LOD was calculated by injecting 0.07µg/ml epigallocatechin 3 gallate three times into a plasma sample and comparing the chromatograms to blank plasma [36]. The LOD was calculated by taking 3.3 times of standard deviation of the response (σ) and the slop of the calibration curve (S)

$$LOD = 3.3\ \sigma / S$$

4.7.6. Limit of quantification

The lowest analyte concentration must be calculated precisely and correctly in a sample. Therefore, three triplicates of a blank plasma sample were compared to three spiked epigallocatechin 3 gallate in plasma at the LLOQ level. Limit of quantification is must often defined as 10 times the standard deviation (σ) of the response and slop (S) of the calibration curve [35-36].

$$LOQ = 10\ \sigma / S$$

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4.7.7. Ruggedness

It refers to the method's ability to reproduce results in real-world conditions. The following three requirements were investigated. First, in the same laboratory, two distinct operators, two columns of the same type and manufacturer, and other reagent and solvent supplies were all tested. For a ruggedness study, epigallocatechin 3 gallate was spiked in rat plasma at two quality control concentration levels (LQC- 5 µg/ml and HQC-25 µg/ml) [30-32, 9].

4.7.8. Robustness

The ability of a method to remain unaffected by minor but deliberate changes in procedure parameters is referred to as robustness. The effect of changing essential parameters in the procedure on peak retention time and concentration was measured through peak area. The organic mobile phase composition of ±5%, the temperature of ±10%, and the flow rate of ±10% were all adjusted systematically, and the chromatograms were compared to the typical chromatographic technique settings. To assess robustness, epigallocatechin 3 gallate was injected in rat plasma at two quality control concentration levels (HQC-25 µg/ml and LQC-5 µg/ml) [35-37, 9].

4.7.9. Stability study of epigallocatechin 3 gallate in rat plasma

LQC and HQC samples were stored at room temperature (30°c) for 6 hours before the inspection to determine their short-term stability. After 10 days of storage at room temperature, the LQC and HQC samples were assessed for long-term stability (30°c). The auto sampler's strength was tested by keeping LQC and HQC samples in the tray for 24 hours at 5 degrees Celsius. Benchtop stability of LQC and HQC was studied by keeping samples at room temperature. These samples were assessed against freshly prepared LQC and HQC concentrations. As part of the freeze-thaw experiment, LQC and HQC samples were taken out of the deep freezer at regular intervals, thawed at room temperature, and stored outside for 1 hour. Before being tested at -30°C, the samples were frozen for three freeze-thaw cycles. Epigallocatechin 3 gallate plasma stability was measured at LQC and HQC levels and compared to the normal epigallocatechin 3 gallate amount [32-37].

4.8. Statistical analysis

GraphPad Prism 8.0.1 was used to validate the findings during statistical analysis.

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REFERENCES

[1] Bartosikova L, Necas J. Epigallocatechin gallate: a review. Vet Med. 2018; 63(10):443–467. http://dx.doi.org/10.17221/31/2018-vetmed
[2] Sunanta W, Kate G, Chartchai K, Thanawat P, Sutasinee A, Chalermpong S. Development of HPLC method for catechins and related compounds determination and standardization in Miang (Traditional Lanna Fermented Tea Leaf in Northern Thailand). Molecules. 2021; 26(19):6052. https://doi.org/10.3390/molecules26196052
[3] Gonzalez-Alfonso JL, Peñalver P, Ballesteros AO, Morales JC, Plou FJ. Effect of α-Glucosylation on the stability, antioxidant properties, toxicity, and neuroprotective activity of (–)-Epigallocatechin Gallate. Front Nutr. 2019;6:30. http://dx.doi.org/10.3389/fnut.2019.00030
[4] Nair HH, Alex VV, Anto RJ. Significance of nutraceuticals in cancer therapy. Evolutionary Diversity As A Source For Anticancer Molecules. 2021;309–321. http://dx.doi.org/10.1016/b978-0-12-821710-8.00014-x
[5] Khan N, Mukhtar H. Tea polyphenols in promotion of human health. Nutrients. 2018;11(1):39. http://dx.doi.org/10.3390/nu11010039
[6] Ungarala R, Sinha SN, Sunder RS. Ultra high-Performance Liquid Chromatography (UHPLC) method development and validation for the identification of oxidized product of Epigallocatechin-3-Gallate (EGCG). J Chromatogr Sci. 2022; 61(2): 140-150. http://dx.doi.org/10.1093/chromsci/bmac028

[7] Fernando CD, Soysa P. Simple isocratic method for simultaneous determination of caffeine and catechins in tea products by HPLC. Springerplus. 2016;5(1):970. http://dx.doi.org/10.1186/s40064-016-2672-9.
[8] Avadhani KS, Amirthalingam M, Reddy MS, Udupa N, Mutalik S. Development and validation of RP-HPLC Method for estimation of epigallocatechin -3-gallate (EGCG) in lipid based nanoformulations. Res J Pharm Technol. 2016;9(6):725. http://dx.doi.org/10.5958/0974-360x.2016.00138.4
[9] Snyder LR, Kirkland JJ, Glajch JL. Practical HPLC Method development, second ed., A Wiley Interscience Publication, 1997.
[10] Mirasoli M, Gotti R, Di Fusco M, Leoni A, Colliva C, Roda A. Electronic nose and chiral-capillary electrophoresis in evaluation of the quality changes in commercial green tea leaves during a long-term storage. Talanta. 2014;129:32-38. https://doi.org/10.1016/j.talanta.2014.04.044
[11] El-Hady DA, El-Maali NA. Determination of catechin isomers in human plasma subsequent to green tea ingestion using chiral capillary electrophoresis with a high-sensitivity cell. Talanta. 2008; 76: 138–145. https://doi.org/10.1016/j.talanta.2008.02.026
[12] Lee LS, Kim SH, Kim YB, Kim YC. Quantitative analysis of major constituents in green tea with different plucking periods and their antioxidant activity. Molecules. 2014;19(7):9173-9186. https://doi.org/10.3390/molecules19079173
[13] El-Kayal M.O, Sayed M.N, Mortada N.D, Elkheshen S. Development and validation of a simple and rapid UPLC method for the in-vitro estimation of (-)-epigallocatechin-3-gallate in lipid-based formulations. Eur J Chem. 2018; 9(1):7–12. https://doi.org/10.5155/eurjchem.9.1.7-12.1661
[14] Park JE, Kim TE, Shin KH. Quantitative analysis of four catechins from green tea extract in human plasma using ultra-performance liquid chromatography-tandem mass spectrometry for pharmacokinetic studies. Molecules. 2018;23(4):984. https://doi.org/10.3390/molecules23040984
[15] Pan HB, Zhang D, Li B, Wu YY, Tu YY. A Rapid UPLC method for simultaneous analysis of caffeine and 13 index polyphenols in black tea. J Chromatogr Sci. 2017;55(5):495-496.https://doi.org/10.1093/chromsci/bmw197
[16] Ramakrishna UV, Shyam SR, Kumar KR, Narayan Sinha S. Method development and validation for rapid identification of epigallocatechin gallate using ultra-high performance liquid chromatography. PLoS One. 2020;15(1):e0227569. http://dx.doi.org/10.1371/journal.pone.0227569
[17] Tamilselvi N, Sinha H, Visakh D, Vanathi P. Bio-analytical method development and validation for the estimation of clotrimazole in human plasma by RP-HPLC method. Res J Pharm Technol. 2016;9(6):671. http://dx.doi.org/10.5958/0974-360x.2016.00126.8
[18] Pongsuwan W, Bamba T, Harada K, Yonetani T, Kobayashi A, Fukusaki E. High-throughput technique for comprehensive analysis of Japanese green tea quality assessment using ultra-performance liquid chromatography with time-of-flight mass spectrometry (UPLC/TOF MS). J Agric Food Chem. 2008;56(22):10705-10708. https://doi.org/10.1021/jf8018003
[19] Wu Z, Medlicott NJ, Razzak M, Tucker IG. Development and optimization of a rapid HPLC method for analysis of ricobendazole and albendazole sulfone in sheep plasma. J Pharm Biomed Anal. 2005; 39(1-2): 225-232. https://doi.org/10.1016/j.jpba.2005.03.010
[20] Hanpitakpong W, Banmairuroi V, Kamanikom B, Choemung A, Na-Bangchang K. A high-performance liquid chromatographic method for determination of praziquantel in plasma. J Pharm Biomed Anal. 2004; 36(4): 871-876. https://doi.org/10.1016/j.jpba.2004.07.043
[21] Dewani A, Dabhade S, Bakal R, Gadewar C, Chandewar A, Patra S. Development and validation of a novel RP-HPLC method for simultaneous determination of paracetamol, phenylephrine hydrochloride, caffeine, cetirizine and nimesulide in tablet formulation. Arab J Chem. 2015; 8(4): 591-598. https://doi.org/10.1016/j.arabjc.2013.09.040
[22] Emami J, Ghassami N, Ahmadi F. Development and validation of a new HPLC method for determination of lamotrigine and related compounds in tablet formulations. J Pharm Biomed Anal. 2006;40(4):999-1005. https://doi.org/10.1016/j.jpba.2005.07.045
[23] Gandla K, Lalitha R, Bommakanti S, Suthakaran R, Pallavi K. Development and validation of RP-HPLC method for simultaneous estimation of albendazole and praziqantel in tablet dosage form. Asian J Pharm Anal. 2015; 5(3):115-118. https://doi.org/10.5958/2231-5675.2015.00018.6
[24] Sagirli O, Onal A, Toker S, Şensoy D. Simultaneous HPLC analysis of olmesartan and hydrochlorothiazide in combined tablets and in vitro dissolution studies. Chromatographia. 2007; 66(3): 213-218. https://doi.org/10.1365/s10337-007-0304-9
[25] Gültekin Y, Öztürk N, Filazi A, Deniz A, Korkmaz Ç, Pezik E, Barın G, Vural İ. Simultaneous determination of albendazole sulfoxide and praziquantel from PLGA nanoparticles and validation of new HPLC method. J Res Pharm. 2022; 26(6): 1608-1618. https://doi.org/10.29228/jrp.252
[26] Tatar E, Gökhan A, Küçükgüzel İ. Development and validation of a RP-HPLC method for quality control of oxantel pamoate, pyrantel pamoat and praziquantel in tablets. Marmara Pharm J. 2015; 19(1): 27-35. https://doi.org/10.12991/mpj.2015198607
[27] Ashish A , Manoj S. Bioanalytical method development and validation for estimation of daunorubicin and cytarabine in blood plasma by using RP-HPLC. J Drug Deliv Ther. 2019; 9(4):341-347. http://dx.doi.org/10.22270/jddt.v9i4.3061
[28] Babu R, Rao A. L, Rao J. V. Bioanalytical method development and validation for simultaneous estimation of paracetamol and cefixime by using RP-HPLC. Orient J Chem. 2016;32(1):701-707. http://dx.doi.org/10.13005/ojc/320178

[29] Satyavert, Gupta S, Nair AB, Attimarad M. Development and validation of bioanalytical method for the determination of hydrazinocurcumin in rat plasma and organs by HPLC-UV. J Chromatogr B Analyt Technol Biomed Life Sci. 2020;1156:122310. https://doi.org/10.1016/j.jchromb.2020.122310
[30] Bhandari R, Kuhad A, Paliwal JK. Development of a new, sensitive, and robust analytical and bio-analytical RP-HPLC method for in-vitro and in-vivo quantification of naringenin in polymeric nanocarriers. J Anal Sci Technol . 2019;10:11. https://doi.org/10.1186/s40543-019-0169-1
[31] Budideti KKR, Kothapalli BCS and Chinnala KM. Bioanalytical method development and validation for the simultaneous determination of vildagliptin and telmisartan in rabbit plasma using RP-HPLC. J Pharm Res Int. 2021; 33(1): 76-86. https://doi.org/10.9734/jpri/2021/v33i131141
[32] Ware AL, Pekamwar SS. Development and validation of bioanalytical RP-HPLC method for determination of valsartan in bulk drug and formulation. Indo Am J Pharm Res. 2020; 11(12): 1455-1473.
[33] Nimje H, Chavan R, Pawar S, Deodhar M. Development and validation of stability-indicating RP-HPLC method for rivaroxaban in tablet dosage form. J Res Pharm. 2022; 26(6): 1703-1712. http://dx.doi.org/10.29228/jrp.261
[34] Veni GK , Ajitha A, Abbulu K. Bioanalytical method development and validation of lenvatinib by RP-HPLC method. IJPSR. 2020;11(7): 3313-3319. https://doi.org/10.26452/ijrps.v10i2.534
[35] Fatema M, Suriya S, Satyajit R, Farhana A, Shammi A, MD. Hossain S. Bioanalytical method validation of esomeprazole by high performance liquid chromatography with PDA detection. Acta Chromatographica. 2021;33(2):120–126. https://doi.org/10.1556/1326.2020.00769
[36] Kanala K, T Hwisa N, Chandu BR, Katakam P, Khagga M, Challa BR, Khagga B. Bioanalytical method development and validation of milnacipran in rat plasma by LC-MS/MS detection and its application to a pharmacokinetic study. J Pharm Anal. 2013 ;3(6):481-488. http://dx.doi.org/10.1016/j.jpha.2013.03.009
[37] Delma D, Anu B, Eena J, Aneesh TP. Bioanalytical method development and validation of Ticagrelor by RP-HPLC. Int J App Pharm. 2017;9(3):51-54. http://dx.doi.org/10.22159/ijap.2017v9i3.17452